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Transcription in Isolated Wheat Nuclei

I. ISOLATION OF NUCLEI AND ELIMINATION OF ENDOGENOUS RIBONUCLEASE ACTIVITY[1]

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ABSTRACT

Nuclei isolated from embryos of wheat (var. Yamhill) incorporated [^{3}H]UTP into a trichloroacetic acid-insoluble product linearly for 60 minutes. When the RNA synthesized *in vitro* was analyzed on a sucrose gradient, the amount of RNA in the 4S region increased with longer incubation times. These data and the absence of higher molecular weight RNA of specific size classes in our work (and previously published reports) suggested that nuclear fractions from plant tissue contained active nucleases. This was confirmed when wheat nuclei were mixed with [^{3}H]yeast RNA (4, 18, 26S). All of the radioactive yeast RNA was degraded within 30 minutes to species sedimenting between 4 and 10S. The inclusion of high salt (125 millimolar $(NH_4)_2SO_4$, 100 millimolar KCl), EGTA, and exogenous RNA or DNA reduced but did not eliminate endogenous RNase activity. Wheat embryo nuclei were further purified by centrifugation on a gradient of a polyvinylpyrrolidone-coated colloidal silica suspension (Percoll). These nuclei were ellipsoidal, free of cytoplasmic material, and lacked endogenous nuclease activity when assayed with [^{3}H]yeast RNA. Sucrose gradients were not as effective as Percoll gradients in purifying nuclei free of RNase activity. The Percoll method of isolating nuclei and the RNase assay reported here will be useful in isolating plant nuclei that are capable of synthesizing distinct RNA species *in vitro*.

The mechanism by which gene transcription and other nuclear events are controlled by factors in the cytoplasm and outside the cell are poorly understood. One approach to the study of specific interactions between nucleus and cytoplasm requires the isolation of active nuclei capable of initiating and completing the synthesis of intact RNA molecules. In such an *in vitro* transcription system, the effect of cytoplasmic components on gene expression can be ascertained. Although RNA synthesis has been studied in isolated plant nuclei by measuring the incorporation of precursors into an undefined product, there have been few reports on the size characterization of the RNA synthesis *in vitro* (7, 15, 17). In a recent paper (15), RNA synthesized by maize nuclei was heterodisperse with little RNA synthesized in size classes greater than 18S. In our preliminary studies on the characterization of RNA synthesized in isolated wheat nuclei, we did not detect the synthesis of RNA greater than 20S. One possible cause of our inability to detect specific high mol wt classes of RNA in plant nuclei is the presence of endogenous nucleases. This report verifies that isolated wheat nuclei contain active RNases. An assay is described to detect the presence of RNase and a method is outlined to eliminate RNase contamination in isolated wheat nuclei so that large mol wt RNA can be recovered.

MATERIALS AND METHODS

Chemicals. Ultrapure sucrose and SDS were purchased from Bio-Rad, Percoll from Pharmacia, and the nylon meshes from Henry Simon Ltd., Cheshire, England. Isotopes were obtained from New England Nuclear and all other biochemicals were purchased from Sigma. Solutions were filter-sterilized or autoclaved, and glassware was heated in 1% (w/v) sodium lauryl sulfate for 15 min at 60 C and rinsed with sterile distilled H_2O prior to use.

Preparation of Plant Material. Wheat embryos were mechanically isolated from dry, mature wheat seeds (*Triticum aestivum* L., var. Yamhill) using a combination of blending and sieving followed by separation on a sucrose gradient (unpublished results). The whole, intact embryo fraction was imbibed in germination medium (3) containing penicillin G (200 μg/ml) and streptomycin sulfate (100 μg/ml) for 3 h at 27 C.

Isolation of Nuclei. Three methods were used to isolate nuclei from purified wheat embryos. All procedures were done at 4 C. In the first method (method I) wheat embryos were homogenized (tissue to buffer, 1:2) for 30 s in a chilled mortar and pestle in a modified Honda buffer (5) containing 0.44 M sucrose, 2.5% (w/v) Ficoll (mol wt 400,000), 5.0% (w/v) Dextran 40, 25 mM Tris-HCl (pH 7.6), 10 mM $MgCl_2$, 10 mM β-mercaptoethanol, and 0.5% (v/v) Triton X-100. After homogenization, 5 volumes of buffer were added and the homogenate was sequentially filtered through four layers of cheesecloth, one layer of Miracloth, and two nylon meshes (80 and 61 μm). The filtrate was centrifuged at 5,850*g* for 5 min and the supernatant was discarded. The nuclear pellet was gently suspended in NRB[4] containing 50 mM Tris-HCl (pH 7.8), 5 mM $MgCl_2$, 10 mM β-mercaptoethanol and 20% glycerol. These nuclei will be referred to as the crude nuclear pellet or nuclei prepared by method I.

The second nuclear isolation procedure (method II) was identical to the first with three exceptions: (*a*) nuclei were homogenized in Honda containing 2 mM spermine (8); (*b*) the nuclear pellet was dissolved in Honda without spermine; and (*c*) the nuclear suspension was more extensively purified by centrifugation in a discontinuous gradient of Percoll (11). The Percoll gradient contained 5-ml layers of 40, 60, 80% (v/v) Percoll on a 5-ml layer of 2 M sucrose cushion. The Percoll contained 0.44 M sucrose, 25 mM Tris-HCl (pH 7.5), and 10 mM $MgCl_2$. The gradients were centrifuged at 4,080*g* in a Sorvall HB4 swinging bucket rotor for 30 min. Most of the nuclei banded in the 80% Percoll, just above the 2 M sucrose cushion. They were removed with a Pasteur pipette,

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[4] Abbreviation: NRB: nuclear resuspension buffer.

washed two times with 10 ml Honda (without spermine) to remove Percoll, and centrifuged again at 5,860*g* for 5 min. The nuclear pellet was washed a third time with NRB. These nuclei were then resuspended in NRB and could be stored at −80 C for several months without loss of activity.

For the third nuclear preparation method (method III), the crude nuclear pellet (from method I) was layered on a discontinuous sucrose gradient. The sucrose gradient contained 5-ml layers of 0.5, 1.0, 2.0, and 3.0 M sucrose dissolved in a solution of 25 mM Tris-HCl (pH 7.5) and 10 mM $MgCl_2$. The gradients were centrifuged as described for method II. Most of the nuclei banded at the interphase between 1 and 2 M sucrose. The nuclei were washed in Honda and NRB as described in method II.

Isolation of [^{3}H]Yeast RNA. Transfer and ribosomal RNA were isolated from yeast cells (strain AP-1) (6). They were grown for 24 h at 30 C in the presence of 10 μCi/ml [^{3}H]adenine (134 mCi/mmol). The label was chased for 4 h at 30 C by washing yeast cells and resuspending in yeast extract-peptone-dextrose medium. Cells were disrupted according to Lipinski *et al.* (9) in lysing buffer (40 mM Tris-HCl [pH 7.5], 40 mM KCl, and 10 mM $MgCl_2$). RNA was extracted as described by Curiale and Mills (4). Transfer and ribosomal RNA were separated on 35-ml sucrose gradient (10–30% in 0.1 M NaCl, 10 mM Tris-HCl (pH 7.5), and 1.0 mM EDTA) at 25,000*g* for 24 h. Gradients were fractionated and those containing 4, 18, and 26S RNA were pooled separately and precipitated with 2 volumes of 95% ethanol. The RNA pellets were collected by centrifugation, dried with N_2, dissolved in sterile-distilled H_2O and stored at −80 C. The specific radioactivity of the 4, 18, and 26S was 22,055, 22,659, and 10,893 cpm/μg respectively.

Assay for Endogenous Ribonuclease Activity. The effect of endogenous nuclease activity on the size distribution of [^{3}H]yeast RNA was tested by incubating nuclei, and yeast [^{3}H]RNA in a volume of 60 μl. In some cases 5 mM EGTA, 100 μg RNA, 100 mM KCl, 125 mM $(NH_4)_2SO_4$ or 20 μg sheared, single-stranded DNA was added to the reaction. After incubation for 30 min, the nuclei were pelleted and the supernatant was layered on 10–40% sucrose gradient. Gradients were centrifuged in a SW 60.1 rotor at about 250,000*g* for 12 h at 4 C and fractionated with an ISCO model 183 density gradient fractionator. The *A* at 254 nm was monitored with an ISCO UA4 analyzer and was recorded with an Omnigraphic Recorder 3000 (Houston Inst.).

Fractions (100 μl) were collected from the gradients and 50-μl aliquots were spotted on filter paper discs (Whatman 3MM, 23 mm). Filters were dried, placed in 5 ml of toluene-Omnifluor (New England Nuclear) and counted in a Packard model 2405 liquid scintillation spectrometer. In this system the counting efficiency for [^{3}H] on filter paper discs was 22%.

RESULTS

Wheat embryo nuclei isolated by method I appeared ellipsoidal and relatively free of cytoplasm. When transcription in these nuclei was assayed by incorporation of [^{3}H]UTP into trichloroacetic acid-insoluble products, the activity increased linearly for 60 min. There was no indication from these data of nuclease contamination. However, when the RNA was isolated from the *in vitro* transcription mixture at 15, 30, and 60 min and analyzed on sucrose gradients there appeared to be an increase in the amount of RNA sedimenting in the 4S region with longer incubation times. The sucrose gradient patterns suggested that these nuclei may be contaminated with nucleases.

To test for endogenous RNase activity, nuclei were incubated with [^{3}H]RNA from yeast. Figure 1A shows the distribution of undegraded yeast RNA into the characteristic 26, 18, and 4S components. When this RNA was incubated with wheat embryo nuclei, most of the yeast RNA was cleaved into products sedimenting in a broad peak near the 4S region (Fig. 1B). The distribution of yeast RNA shown in Figure 1B was almost identical to the pattern of [^{3}H]RNA synthesized *in vitro* by wheat nuclei. The addition of components which were shown to reduce nuclease activity in other systems, such as sheared, single-stranded DNA, high salt (350 mM monovalent cation), and EGTA, did not eliminate this nuclease activity (Fig. 1C).

In an attempt to remove the RNase, we purified the nuclei prepared by method I on a discontinuous gradient of Percoll (11), a PVP coated silica suspension (Fig. 2A). When the nuclei were removed from the 80% Percoll region and washed, the nuclei were intact and free of cytoplasmic contamination when viewed with Nomarski optics (Fig. 2B) or when stained with methyl green-

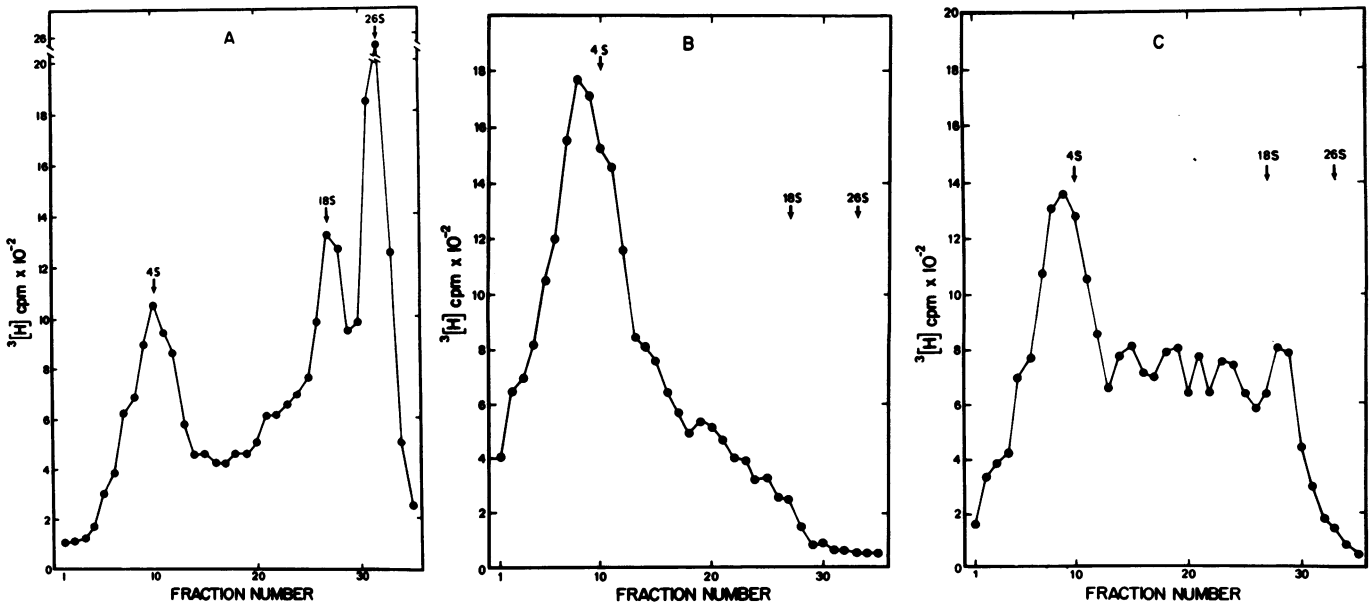

FIG. 1. Sucrose gradient analysis of [^{3}H]yeast RNA when mixed with wheat embryo nuclei. Yeast RNA without nuclei added (A), with crude nuclei (10 μl; 3.1 × 10^5 nuclei) prepared by method I (B), with crude nuclei (10 μl; 3 × 10^5 nuclei) in the presence of 5 mM EGTA, 20 μg sheared, single-stranded DNA, 125 mM $(NH_4)_2SO_4$, and 100 mM KCl (C).

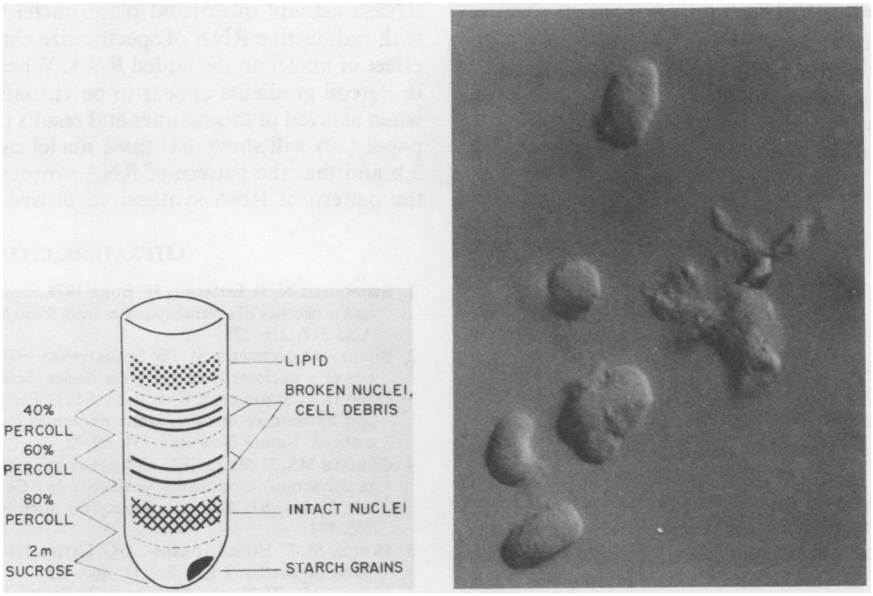

FIG. 2. Schematic representation of a Percoll discontinuous gradient and banding of cellular components (A). Photomicrograph of wheat embryo nuclei isolated from 80% Percoll region of the gradient taken with Nomarski optics (B) (× 750).

pyronin B (12). When these purified nuclei were incubated with [^{3}H]yeast RNA for 30 min (in the absence of RNase inhibitors) there was little degradation of yeast RNA (Fig. 3). In addition, the nuclei were intact when observed after the 30-min incubation.

To determine if sucrose was as effective as Percoll in removing RNase activity from the nuclei, discontinuous sucrose gradients were tested. Nuclei purified on sucrose gradients were more contaminated with cytoplasm, 40% less active in the *in vitro* transcription reaction and exhibited more RNase activity than nuclei purified in Percoll. The data in Table I show that 25% of the total yeast RNA on the control gradient (without nuclei added) sediments in the top of the gradient (fractions 1–15), while 75% of the radioactivity sediments in the region where RNA is greater than 16S (fractions 16–35). An almost identical distribution of counts was observed when nuclei purified in Percoll were mixed with [^{3}H]yeast RNA. When [^{3}H]yeast RNA was incubated with nuclei isolated in sucrose, the distribution of radioactivity more closely resembled the pattern obtained when a crude nuclear pellet was incubated with the yeast RNA (Table I). In addition, the sucrose gradient nuclei were no longer intact after the 30-min incubation. Sucrose gradient centrifugation did not appear to be as effective as Percoll gradient centrifugation in isolating nuclei free from endogenous RNase activity.

DISCUSSION

Prior to and during this study, only three reports described the size of RNA synthesized from nuclei isolated from plants: Johri and Varner (7) using pea shoots, Wilson and Bennett (17) using pea leaves, and Slater *et al.* (15) using corn shoots. In all three cases, little if any RNA was detected in a size class larger than 20S. Most of the RNA had S values between 4 and 10 and no discrete size classes were demonstrated. One possible cause of the absence of high mol wt RNA synthesis was the presence of endonucleases which have been shown to be associated with chromatin from bean seeds (14) and wheat seedlings (13), as well as in nuclei from rye germ (2). Root tissue from *Vicia faba* also contained nucleases (1) and when Trapy *et al.* (16) attempted to isolate rRNA from this tissue their rRNA products sedimented in the 4–10 region of a sucrose gradient. A specific endonuclease was implicated in these results.

The major problem we faced in isolating nuclei which would

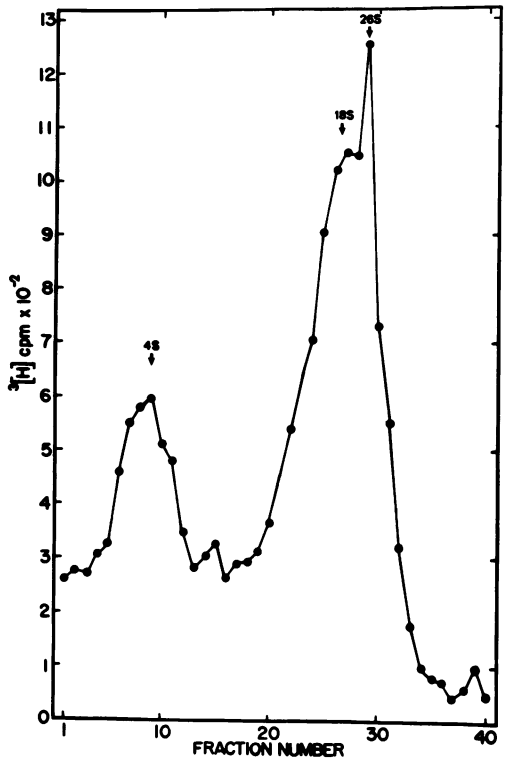

FIG. 3. Sucrose gradient analysis of [^{3}H]yeast RNA when mixed with Percoll purified wheat embryo nuclei prepared by method II.

synthesize high mol wt RNA also appeared to be RNases of an endonucleolytic type. We found that wheat embryo nuclei have RNase activity as evidenced by their ability to degrade exogenously added radioactive yeast RNA (26, 18, 4S). Although acid-soluble counts (*i.e.* RNase activity) were reduced by 60% when high salt, EGTA, and tRNA or DNA was added to the nuclei/yeast RNA mixture (unpublished data), the yeast RNA from this mixture was still degraded to a 4–10S peak when analyzed on a sucrose gradient (Fig. 1C). Patterns of RNA synthesized *in vitro* by nuclei from corn (15) and pea (7) showed no rRNA species or

Table I. *Distribution of Yeast [^{3}H]RNA in a 10–40% Sucrose Gradient*

[^{3}H]Yeast RNA was incubated for 30 min at 30 C: (1) without nuclei; (2) with Percoll-purified nuclei (method II); (3) with crude nuclei (method I); or (4) with sucrose-purified nuclei (method III). The nuclei were isolated and the RNA separated on sucrose gradients. The data are reported as the per cent of total counts sedimenting in the 0–16S region (fractions 1–15) and from 17S to the bottom of the gradient (fractions 16–35).

	Total Radioactivity	
	0–16S	>17S
	% cpm	
(1) Yeast RNA nuclei	25	75
(2) Yeast RNA + Percoll nuclei	33	67
(3) Yeast RNA + crude nuclei	73	27
(4) Yeast RNA + sucrose nuclei	68	32

RNA of higher mol wt, suggesting the presence of nucleases. Although no gradients were shown, Wilson and Bennett (17) mentioned that they had active nucleases in their pea nuclei preparations since no precursor to rRNA was detected.

The nuclease activity could result from cytoplasmic RNase adsorbed to nuclei, or once nuclei are removed from their cytoplasmic environment the regulation of nuclear RNase activity is lost. The likelihood of contamination by exogenous RNase is unlikely in our results since precautions were taken to eliminate its activity (*e.g.* SDS-treated glassware and sterilized solutions) and yeast 4, 18, and 26S RNA were isolated and separated without degradation.

We found that wheat embryo nuclei isolated on a discontinuous Percoll gradient were intact, ellipsoidal and free of cytoplasm when stained with methyl-green pyronin B (12). These nuclei contained little if any endogenous nuclease activity when they were incubated with [^{3}H]yeast RNA. We are not certain how the Percoll isolation reduces the endogenous RNase activity: Percoll could bind the nucleases or it could facilitate the separation of cytoplasm containing RNase from the nuclei. We have found that isolation of nuclei on Percoll gradients appears to be more effective in removing nuclease activity than centrifugation on comparable sucrose gradients (Table I).

Although nuclei can incorporate radioactive precursors into acid-insoluble material, considerable RNA degradation can occur and escape detection by this assay. It is important to test for RNase activity in isolated plant nuclei by incubating the nuclei with radioactive RNA of specific size classes and determining the effect of nuclei on the added RNA. Wheat embryo nuclei isolated in Percoll gradients appear to be virtually free of RNase activity when assayed in this manner and results presented in the following paper (10) will show that these nuclei can synthesize RNA up to 3 h and that the pattern of RNA synthesized *in vitro* is similar to the pattern of RNA synthesized *in vivo*.

LITERATURE CITED

1. Beopoulos N, R Esnault, JF Bure 1978 Study on plant RNAases. Isolation and properties of several activities from *Vicia faba* root cells. Biochim Biophys Acta 517: 216–227
2. Boruka-Mankiewicz M, JW Szarkowski 1977 Purification and some properties of a nuclease from rye germ nuclei. Acta Biochim Pol 24: 289–300
3. Chen D, G Schultz, E Katchalski 1977 Early ribosomal RNA transcription and appearance of cytoplasmic ribosomes during germination of the wheat embryo. Nature New Biol 231: 69–72
4. Curiale MS, D Mills 1975 Characterization of pulse-labeled ribonucleic acid in polysomes of sporulating yeast cells. *In* P Gehardt, RN Coslilaw, HL Sadoff, eds, Spores, VI. American Society for Microbiology, East Lansing, Mich, pp 165–171
5. Honda SI, T Hongladarom, GG Laties 1966 A new isolation medium for plant organelles. J Exp Bot 17: 460–472
6. Hopper AK, PT Magee, SK Welch, M Friedman, B Hall 1974 Macromolecule synthesis and breakdown in relation to sporulation and meiosis in yeast. J Bacteriol 119: 219–238
7. Johri MM, J Varner 1967 Enhancement of RNA synthesis in isolated pea nuclei by gibberellic acid. Proc Nat Acad Sci USA 59: 269–276
8. Kaur-Sawhney R, A Altman, AW Galston 1978 Dual mechanisms polyamine-mediated control of ribonuclease activity in oat leaf protoplasts. Plant Physiol 62: 158–160
9. Lipinski C, AJ Ferro, D Mills 1976 Macromolecular synthesis in a mutant of *Saccharomyces cerevisiae* inhibited by *S*-adenosylmethionine. Mol Gen Genet 144: 301–306
10. Luthe DS, RS Quatrano 1980 Transcription in isolated wheat nuclei. II. Characterization of RNA synthesized *in vitro*. Plant Physiol 65: 309–313
11. Pertoft H, TC Laurent, T Laas, L Kagedal 1978 Density gradients prepared from colloidal silica particles coated by polyvinylpyrrolidone (Percoll). Anal Biochem 88: 271–282
12. Rizzo PJ, K Pederson, JH Cherry 1978 Transcription and RNA polymerase stimulatory activity in nuclei isolated from soybean. Plant Sci Lett 12: 133–143
13. Sasaki K, S Sasaki 1976 Low temperature ribonuclease in chromatin of winter wheat seedlings. Biochim Biophys Res Commun 72: 850–858
14. Slater RJ, D Grierson 1977 Ribonucleic acid synthesis by chromatin isolated from *Phaseolus aureus* Roxb. Planta 137: 153–157
15. Slater RJ, MA Venis, D Grierson 1978 Characterization of ribonucleic acid synthesized by nuclei isolated from *Zea mays*. Planta 144: 89–93
16. Trapy G, R Esnault, Y Zuily-Fodil 1975 Conditions d'inactivation de ribonuclease lies aux RNA vegetaux. Plant Sci Lett 4: 257–265
17. Wilson PS, J Bennett 1976 Transcription in nuclei isolated from a higher plant. Biochem Soc Trans 4: 812–813